POISONING AND RECOVERY IN BARNACLES AND MUSSELS [1]

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Introduction

Previous investigations have shown that the prevention of the attachment of organisms to ships' hulls by antifouling paint is related to the rate of solution of the toxic material, and hence, to its concentration in the sea water at the paint's surface (Ketchum, Ferry, Redfield, Burns, 1945). The present investigation was undertaken to test the effectiveness of different metallic salts as poisons for two important types of fouling organisms and to evaluate them under conditions in which the concentration and ionic form of the toxic materials were accurately known. In earlier attempts to test lethal action, supposedly toxic materials have been incorporated in paints applied to test-panels or to the inside surfaces of containers and the degree of fouling resistance noted, but in most cases no adequate measure was made of the concentrations of the toxics which were actually emitted by the paint. In other tests, fouling organisms were placed in containers of sea water to which known amounts of toxics had been added but without adequate knowledge of the ionic transformation of the toxics which ensue in the presence of sea water (Bray, 1919; Edmondson and Ingram, 1939; Orton, 1929–30; and Parker, 1924).

We proposed, therefore, to conduct direct tests of the action of various individual metals in graded concentrations and in different chemical forms, on two important types of fouling organisms; namely, barnacles and mussels. We desired also to investigate the relationship between the concentration of various toxics and the exposure time necessary to kill, in order to ascertain the most efficient concentration of the poison for the destruction of fouling organisms.

A second purpose was to follow the course of poisoning and recovery in barnacles and mussels when copper was used as the poison. It was desired to measure the amount of copper in the tissues and to discover whether death from copper poisoning was brought about specifically by the accumulation of a certain amount of the poison in the tissues or by the entrance of the copper into the body at a certain rate or for a critical period of time. Finally, it was desired to ascertain from how great a dose of copper an animal could recover, and to learn whether, after recovery, the animal was more or less susceptible to subsequent exposures of the toxic.

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Observations on Adult Barnacles and Mussels

The effectiveness of various poisons was investigated first using the barnacle as a test animal, since the barnacle is generally the most serious offender as a fouling organism. Adult barnacles were employed for the majority of the experiments at Woods Hole, but one set of tests was conducted on nauplii which had been discharged in a laboratory tank. Although attachment of the barnacle takes place previous to the adult stage, there is evidence that a poisoned barnacle is less securely attached than a normal animal and may be more easily dislodged. Special interest centers about the poisoning of the cyprid, since this is the attaching stage. In view of the fact that cyprids could not be obtained in sufficient numbers at Woods Hole, experiments on this stage were conducted at Miami Beach, Florida. Mussels, which are of relatively large size, were found advantageous for other tests, especially those involving the copper assay of living tissue.

Source and preparation of material

Certain of the present tests were carried out with *Balanus balanoides* growing on stones obtained near low tide mark at Woods Hole. In later tests *Balanus eburneus* was employed, obtained through the kindness of Dr. Stanley Cobb, from a wharf in Cotuit Harbor. Parts of the wharf were removed and transported to the laboratory tanks. The barnacles were kindly identified by Dr. Richard McLean.

For the experiments with *B. balanoides* the material was collected on the same day that the tests were begun. Single stones bearing 10 to 30 barnacles (ranging from 3 mm. to 10 mm. in diameter) were placed in individual beakers or culture dishes containing from 250 cc. to 500 cc. of sea water with or without toxic. For the work using *B. eburneus,* a stock of animals attached to fragments of wood was maintained in large battery jars provided with running sea water. In preparation for the tests it was necessary to split and saw the pieces of wood into blocks of convenient dimensions. Blocks about 4 cm. to 6 cm. square, each bearing 10 to 20 barnacles ranging from 4 mm. to 20 mm. in diameter, were similarly placed in individual containers. In other experiments larger blocks bearing several hundred barnacles were placed in battery jars of 8 liters capacity or more.

With both species of barnacles it was found that when the sea water was renewed daily, the animals in the individual containers remained in a healthy condition for several weeks. There were evidently enough food particles in the sea water supplied to provide a maintenance diet. Faecal material was produced in abundance but the barnacles grew little, if at all.

The mussels employed were the common species in the Woods Hole region, *Mytilus edulis,* obtained from the dock and float of the Oceanographic Institution. The mussels were placed individually or in batches in culture dishes or large battery jars. The animals began attaching their byssus threads to the bottoms or sides of the containers almost immediately. If the sea water was renewed each day, the mussels continued in healthy condition indefinitely, apparently obtaining sufficient nutriment from the food particles present in the sea water.

Behavior during poisoning

When barnacles were placed in the toxic media, they opened their shells and began raking or filtering the water with their cirri as soon as did the controls in fresh sea water. Ordinarily this took place within a few minutes. If the concentration of the poison was such as to kill the barnacle within two or three days, the animals would begin to show signs of bad condition after about 12 hours. The first symptom was the slowing down of the beat of the cirri and soon thereafter their sweep became reduced in extent. As poisoning progressed, the movement of the cirri became still slower and more curtailed until they opened to only one-half or one-quarter of their usual scope. When the poisoned barnacle stopped filtering, it tended to come to rest with the cirri half extended, in contrast to the healthy animal in which the cirri are drawn completely in and the shell tightly closed during rest. In the poisoned animal, the shell could at first be caused to close by prodding it with a needle. After further poisoning, when no response could be elicited by stimulation with the needle, the barnacle was considered dead. The following symbols are used in the tables to designate these conditions:

++++	normal rapid sweep of cirri
+++	full sweep but slow
++	incomplete sweep
+	open, inactive, but reacts to touch
0	dead

If the barnacle was replaced in fresh sea water (preferably running sea water) within a day of the time it reached the next to last condition (+), it would usually recover completely its full activity after a few days.

In the case of the mussel, the change in condition as poisoning progressed was less easily observed. In the higher concentrations of the toxics, the animals failed to attach themselves to the walls of the containers and tended to remain closed for long periods. When placed in weaker solutions, however, many of the mussels became strongly attached and remained with their shells open to the normal extent to allow the inflow and outflow of water. In either case, after poisoning had progressed to a certain point, the valves were found gaping wide open. When this condition was first reached, the animal responded to prodding by closing slowly. A day later, or less, no response would follow such stimulation and the animal was considered dead. Once the mussel reached the stage in which it gaped open more than normal, though it might still react, it would not recover when placed in fresh sea water. In fact, mussels were frequently observed to die many days after having been transferred from the toxic solution into fresh sea water although outwardly they appeared to be in normal condition in the interim.

Toxicity of various metallic salts to barnacles

The toxicity of the following metals was investigated: copper, mercury, silver, and zinc. The last three of these was tested as the ions which resulted from the introduction into the sea water of $HgCl_2$, $AgSO_4$, and $Zn(NO_3)_2$ respectively. The copper, on the other hand, was tested not only in the form of basic cupric carbonate, but also as cupric citrate, cupric tartrate, cupric salicylate, and cupric para amino-

benzoate. The interest in these other compounds of copper lies partly in the possibility of revealing important differences in toxicity. Of further significance is the fact that these substances are much more soluble in sea water than is basic cupric carbonate. Therefore, when copper is supplied in the form of these more soluble ions, it is possible to establish a high concentration of the toxic in the water adjacent to the surface from which the material is free to dissolve.

Individuals of *Balanus balanoides* were placed in beakers or jars containing graded concentrations of toxics in sea water with a control for each series. Since the media were renewed each day, the danger of significant changes in concentration due to such causes as adsorption was minimized. The minimum concentrations necessary to kill 90 per cent of the barnacles after continuous exposures of two days and five days are indicated in Table I. Among the various forms of copper, the

TABLE I

The concentrations of various metallic salts necessary to kill 90 per cent of adult barnacles after continuous exposures for the indicated periods. Concentrations are expressed as milligrams of metal per liter of sea water

	Toxic	Concentration lethal in 2 days	Concentration lethal in 5 days
		mg./liter	mg./liter
Series I			
	Basic Cu Carbonate	0.35	—
	Basic Cu Carbonate	0.48	—
Balanus balanoides	Cu Citrate	0.60	0.18
from	Cu Citrate	0.60	0.30
Woods Hole harbor	Cu Tartrate	0.58	0.17
	$HgCl_2$	1.0	0.5
	Ag_2SO_4	0.4	0.2
	$Zn(NO_3)_2$	32.	8.0
Series II			
	Basic Cu Carbonate	0.28	0.14
Balanus eburneus	Cu citrate	0.55	0.14
from	Cu salicylate	0.90	0.45
Cotuit harbor	Cu para aminobenzoate	—	0.50

basic cupric carbonate was the most effective but the concentrations of the citrate and tartrate necessary to kill were only slightly greater. The toxicity of silver as silver sulphate was of the same order of magnitude but somewhat greater concentrations of mercury as mercuric chloride were required. The effectiveness of zinc, on the other hand, was very much less than any of the other substances tested (*cf.*, Riley, 1943).

Experiments with *Balanus eburneus* indicated similarly that the lethal action of cupric citrate is somewhat less than that of basic cupric carbonate although the magnitude of the difference is not great. The toxicity of cupric salicylate and cupric aminobenzoate were somewhat less than the citrate.

In view of the fact that the toxicity of cupric citrate was found to be almost as great as that of basic cupric carbonate, and since stock solutions of higher and less

variable concentrations could be maintained, the tests which follow were conducted with the citrate.

Relation between concentration of cupric citrate and killing time

Barnacles

With the higher concentrations of cupric citrate in which killing occurred within a few days, adult barnacles all succumbed at about the same time, but at the lower concentration it was difficult to determine accurately when the barnacles should be considered dead. At still greater dilutions of the toxic, it was found that most of the animals lived on in a feeble condition for two or three weeks or more. Furthermore, there was considerable variation from experiment to experiment, which was no doubt due not only to individual differences in the batches of barnacles, but also

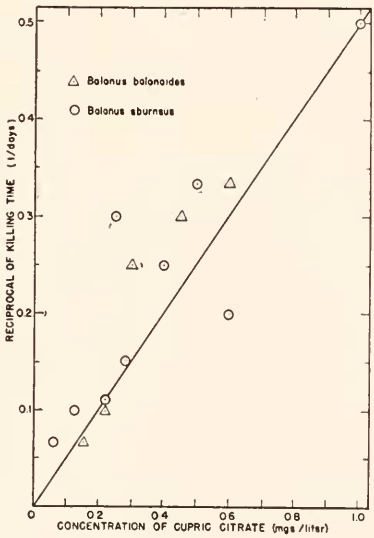

FIGURE 1. The relation between killing time and concentration of toxic for two species of barnacles.

to unavoidable changes in the seasonal condition and in the age of the animals. No consistent trend appeared in relation to the latter conditions except that barnacles in the nauplius stage were killed much more quickly than the adults, as noted below.

For both *B. balanoides* and *B. eburneus,* the killing time ranged between two and five days for concentrations of 0.60 to 1.00 mg. Cu/liter. At 0.22 mg./liter the animals succumbed after ten days. At concentrations of 0.06 mg./liter and less, the animals remained alive for two weeks or more. When the reciprocal of the killing time was plotted against the concentration, as in Figure 1, a roughly linear relation was found. The approximate linearity of this relation indicates that the rate of action of the poison is directly proportional to its concentration.

The results of a series of tests on the poisoning of nauplii, made on the day of their release from the brood pouch, are shown in Table II. They indicate that the

TABLE II

Effect of concentration of cupric citrate on the survival of nauplii of Balanus eburneus. August 25–27, 1942

Concentration mg. Cu/liter	After 22 hrs.	After 29 hrs.	After 48 hrs.
1.00	all dead	—	—
0.50	all dead	—	—
0.25	all dead	—	—
0.13	75% alive	all dead	—
0.06	75% alive	50% alive	1% alive
0.03	100% alive	95% alive	5% alive
Control	100% alive	95% alive	5% alive
Control	100% alive	95% alive	10% alive

nauplii are killed after much shorter exposure than is the case with the adults. The test was not very satisfactory because the controls lived for only about two days.

Mussels

The experiments on the mussel, *Mytilus edulis,* indicate that it is much more sensitive to cupric citrate than the barnacle (Tables III and IV). A half-day exposure to 0.55 mg. Cu/liter was sufficient to kill the three mussels in this concentration. One animal in each of the experiments succumbed following a half-day exposure to a concentration of only 0.14 mg. Cu/liter. With adult barnacles, exposures of two days and five days are required for killing at these concentrations. In these tests, and in many others with mussels, death did not ensue until several days after the animals had been transferred to fresh sea water. In one case the mussel did not die until the tenth day following a half-day exposure to the poison. In most cases the animals showed no outward sign of unhealthy condition in the interim.

TABLE III

Relation of killing time to concentration of cupric citrate in the mussel, Mytilus edulis. In each test three animals (about 3 cm. long) were placed in each toxic solution for the periods indicated and then transferred to fresh sea water. Started August 6, 1942

Concentration mg. Cu/liter	Time in toxic days	Killing time days
0.55	½	4, 8, 10
	1	2, 3, 4
	2	2, 3, 3
	3	2, 3, 3
0.14	½	4, *, *
	1	3, 3, 4
	2	3, 4, 4
	3	3, 4, 4

* Still alive when experiment terminated on August 18.

TABLE IV

Relation of killing time to concentration of cupric citrate in the mussel, Mytilus edulis. In each test two animals (about 3 cm. long) were placed in each toxic solution for the periods indicated and then transferred to fresh sea water. Started August 10, 1942

Concentration mg. Cu/liter	Time in toxic days	Killing time days
0.14	½	5, *
	1	3, 4
0.08	½	*, *
	1	*, *
	2	6, 8
0.04	½	*, *
	1	*, *
	2	*, *
	3	8, *
0.02	1	*, *
	2	*, *
	4	*, *

* Still alive when experiment terminated on August 20.

Accumulation of copper in barnacle tissues during poisoning and recovery

Several series of experiments were undertaken to determine the extent to which copper accumulated in the tissues of the barnacles subjected to solutions of cupric citrate. Since the shell material of the barnacle is several times greater in volume and many times greater in dry weight than the soft tissues, a technique was developed for the copper assay of both constituent parts of the animal separately. The number of barnacles used for a single analysis varied from 15 to 40 with the size and expected copper content. In the earlier experiments, the animals were chiseled free from the substratum, but in so doing all the fluid material was lost, and bits of broken shell were sometimes included with the soft parts. In the later experiments the soft tissues were taken from the shell without disengaging the animals from the substratum by removing the operculum and picking out the soft parts with forceps. The fluid material, which was removed with a pipette, and the tissues were then placed in a weighed Erlenmeyer flask. In the case of mussels, a much smaller number of animals was used. The relatively large amount of sea water within the mantle cavity of the mussel was drained off and discarded before removing the soft parts.

The wet weight of the soft tissues of barnacles and mussels was obtained and the approximate volume of the wet tissue was determined by forcing it into a graduated cylinder.[3] The material was transferred quantitatively to a flask, dried in an oven at 100° C., and the dry weight determined. Enough H_2SO_4 was added to char the material. It was heated to fumes on a hot plate, and concentrated HNO_3 was added dropwise until a colorless solution was obtained. The solution in the flask was transferred quantitatively to a 100 cc. volumetric flask and diluted to the mark. Test for copper was made by the carbamate method (Coulson, 1937). A blank to which the same amount of reagent was added was also carried through the above procedures. In the analysis of barnacle shells, the wet weight of the shells was first

[3] In the early experiments, volume in cubic centimeters alone was determined, but was subsequently found to be very nearly equal to the wet weight in grams.

determined. The shells were then dried in the oven at 100° C. overnight and the dry weight determined. The material was ignited in the muffle furnace at 800°–900° C. for three hours, removed and cooled. Two cubic centimeters of concentrated HCl were added, warming if necessary to dissolve.[4] The remainder of the analysis followed the same procedure as with the soft tissues.

Determinations revealed that the amount of copper in the shell tissue of both normal and poisoned barnacles was quite variable. There was also no consistent change in the copper content of the shells in the case of batches of poisoned barnacles with increasing amounts of copper in the soft tissues. Accordingly, in the later experiments the laborious and rather unsatisfactory copper assay of the shells was omitted.

The copper content of barnacles freshly brought into the laboratory is many times greater than an equal volume or an equal weight of sea water (Table V). Evidently under natural conditions, the barnacle tends to accumulate copper in its tissues to a considerable extent.

TABLE V

Copper content of freshly collected barnacles
Woods Hole sea water contains 0.00001 to 0.00003 mg./cc.

Species	Source	Number	Copper in soft parts			Copper in shells		
			mg.	mg./cc.*		mg.	mg./cc.	
Balanus eburneus	Cotuit, July 28	50	0.0017	0.0014		0.141	0.027	
	Cotuit, July 28	50	0.0027	0.0024		0.097	0.014	
			mg.	mg./g. Wet wt.	mg./g. Dry wt.	mg.	mg./g. Wet wt.	mg./g. Dry wt.
Balanus eburneus	Cotuit, Aug. 20	50	0.015	0.0038	0.008	0.007	0.0003	0.0004
	Cotuit, Aug. 20	40	0.010	0.0019	0.014	—	—	—
	Cotuit, Sept. 12	30	0.016	0.0011	0.016	—	—	—
Balanus balanoides	Woods Hole, July	100	0.029	0.029	0.104	0.069	0.0070	0.0075
	Buzzards Bay	50	0.023	0.027	0.068	0.063	0.0065	0.0067

* May be assumed equivalent to mg./g. wet weight.

In experiments designed to test whether barnacles could accumulate copper in their tissues, it was found that the amount of copper present increased rapidly for each day that the animals remained in the toxic solution. At the end of four days of continuous poisoning in the two concentrations used, all the animals were dead (Blocks A and C, Tables VI and VII). However, in those cases in which the barnacles were transferred to fresh sea water after one or two days, the animals were restored to what appeared to be a healthy condition.[5] Analysis of the tissues of the barnacles which had been replaced in fresh sea water showed that the animals had not only recovered their activity but also had eliminated a certain amount of copper. These barnacles still contained, however, as much as twenty-five times the amount of copper found in their tissues before poisoning began.

[4] If an excess of $CaCO_3$ remained, more acid was added until evolution of CO_2 ceased.

[5] The recovery of Bugula larvae from copper poisoning was reported by Miller and Cupp (1942) and Miller (1946).

TABLE VI

Changes in copper content of soft tissues during poisoning and recovery

Toxic: Cupric citrate, conc. 0.20 mg. Cu/liter
Test Animal: Balanus eburneus
Date: August 3–8, 1942
*Basis: Copper content per cubic centimeter of soft tissue **

Block A		Block B	
Initial copper content	0.010 mg.	Initial copper content	0.010 mg.
After 1 day in toxic	0.034 mg.	After 1 day in toxic	0.034 mg.
After 2 days in toxic	0.070 mg.	After 1 day in sea water	0.033 mg.
After 3 days in toxic	0.093 mg.	After 2 days in sea water	0.030 mg.
After 4 days in toxic	0.112 mg.	After 3 days in sea water	0.035 mg.
		After 4 days in sea water	0.026 mg.
Condition at end—dead		Condition at end—open, inactive but reacts to touch	

* May be assumed equivalent to wet weight.

TABLE VII

Changes in copper content of soft tissues after varying exposures to toxic and after recovery

Toxic: Cupric citrate, conc. 0.35 mg. Cu/liter
Test Animal: Balanus eburneus
Date: August 10–17, 1942
*Basis: Copper content per cubic centimeter of soft tissue **

Block C		Block D		Block E	
Initial Cu content	0.002 mg.	Initial Cu content	0.002 mg.	Initial Cu content	0.002 mg.
After 1 day in toxic	0.024 mg.	After 1 day in toxic	0.024 mg.	After 1 day in toxic	0.024 mg.
After 2 days in toxic	0.073 mg.	After 2 days in toxic	0.073 mg.	After 1 day in sea water	0.029 mg.
After 3 days in toxic	0.089 mg.	After 1 day in sea water	0.056 mg.		
After 4 days in toxic	0.075 mg.	After 9 days in sea water	0.054 mg.	After 6 days in sea water	0.022 mg.
Condition at end—dead.		Condition at end—cirri give incomplete sweep or full sweep but slow.		Condition at end—normal rapid sweep of cirri.	

* May be assumed equivalent to wet weight.

These indications that barnacles can take up relatively large quantities of copper without being killed and can eliminate the poison to a certain extent were confirmed by more elaborate experiments which were designed primarily to test the effects of the toxic and of different periods of exposure and of recovery (Tables VIII to XI).[6] These experiments demonstrated that barnacles took up copper

[6] In these experiments the dry weight of the soft tissues to be analyzed was determined and the copper content stated on the more accurate basis of milligrams of copper per gram of dry weight. The dry weight for the soft parts of the barnacle was found to be about 5 per cent to 20 per cent of the wet weight. Since this group of experiments was conducted during late summer and autumn, there exists the possibility that the results may not be strictly comparable with the earlier work. The barnacles tested were necessarily older and larger, and were no doubt in a different physiological condition. It was noted that the animals became less active as the season progressed, and by late autumn they tended to remain closed for long periods. This last fact alone would be expected to cause an important difference in the rate at which toxic was absorbed. In general, the animals tended to survive longer in high concentrations of toxic in the autumn than they had in the summer.

TABLE VIII

Comparison of changes in copper content of soft tissues during continuous exposure to low concentration of toxic and intermittent exposure to higher concentration

Toxic: Cupric citrate, Block F: 0.14 mg. Cu/l.; Block G: 0.22 mg. Cu/l.
Test Animal: Balanus eburneus
Date: August 24 to September 28, 1942
Basis: Copper content per gram of dry weight of soft tissue
Exposure times indicated for toxic are cumulative totals

Block F		Block G	
Initial copper content	0.014 mg.	Initial copper content	0.008 mg.
After 1 day in toxic	0.05 mg.	After 1 day in toxic	0.037 mg.
After 2 days in toxic	0.08 mg.	(Replaced in sea water for 1 day)	—
After 7 days in toxic	0.33 mg.	After 2 days in toxic	0.095 mg.
After 15 days in toxic	1.09 mg.	(Replaced in sea water for 1 day)	—
(Replaced in fresh sea water)		After 3 days in toxic	0.18 mg.
After 18 days in sea water	0.39 mg.	(Replaced in sea water for 6 days)	—
		After 4 days in toxic	0.23 mg. (0.19 mg.)*
		(Replaced in sea water for 7 days)	0.17 mg.
		After 7 days in toxic	0.48 mg.
		(Replaced in sea water for 16 days)	0.51 mg.
Condition at end—full but slow sweep of cirri		Condition at end—full but slow sweep of cirri	

* Batch of dead barnacles.

faster from the more concentrated toxic media. With the slower rate of absorption at the lower concentrations, the animals appeared to withstand higher amounts of copper in their tissues. Thus the highest copper content observed for living barnacles, namely, 1.09 mg. per g. dry weight, was found after fifteen days in 0.14 mg. Cu/liter cupric citrate (Table VIII, Block F). In cases in which large amounts of copper were absorbed the barnacles tended to live longer and to remain in better condition when periods in sea water were alternated with periods in the toxic medium.

In cases where the copper content was determined for the dead barnacles, which were found among the living individuals in increasing number as the experiment progressed, it was almost invariably true that the amount of copper present was less than that for the living specimens from the same block. This observation suggests that the entrance of copper and its fixation in the tissues of the barnacle is not simply a mechanical process of diffusion or adsorption but is a process which is influenced in some way by the metabolic reactions of the living organisms.

Due to the facts considered above, it is not possible to arrive at any definite value for an absolute amount of copper which must be absorbed by the barnacle to kill it, nor at any rate of absorption above which death will ensue. The results suggest that killing may not be due to the destruction of some vital substance or process by the direct action of the copper as much as to a general depressing action of the toxic on some function, such as the feeding reaction, to the extent that death eventually follows.

TABLE IX

Comparison of changes in copper content of soft tissues during continuous and intermittent exposures to high concentration of toxic and continuous exposure to lower concentration

Toxic: Block H—Cupric citrate 0.75 mg. Cu/l. Continuous
Block I—Cupric citrate 0.35 mg. Cu/l. Continuous
Block J—Cupric citrate 0.75 mg. Cu/l. Alternating daily with sea water
Test Animal: Balanus eburneus
Date: September 21 to October 14, 1942
Basis: Copper content per gram of dry weight of soft tissue

Block H		Block I	
Initial Cu content	0.016 mg.	Initial Cu content	0.016 mg.
After 2 days in toxic	0.17 mg.	After 3 days in toxic	0.13 mg.
After 4 days in toxic	0.30 mg.	After 6 days in toxic	0.25 mg.
After 6 days in toxic	0.38 mg.	After 9 days in toxic	0.32 mg.
After 7 days in toxic	(0.33 mg.)*	After 11 days in toxic	0.76 mg.
After 8 days in toxic	0.48 mg.	After 14 days in toxic	(0.39 mg.)*
After 11 days in toxic	(0.84 mg.)*	After 14 days in toxic	0.49 mg.
		After 18 days in toxic	(0.43 mg.)*
		After 18 days in toxic	0.60 mg.

Block J

Initial Cu content	0.016 mg.
After 2 days in toxic, 1 day in sea water	0.16 mg.
After 4 days in toxic, 3 days in sea water	0.13 mg.
After 6 days in toxic, 6 days in sea water	0.31 mg.
After 8 days in toxic, 7 days in sea water	0.42 mg.
After 8 days in toxic, 8 days in sea water	0.25 mg.
After 10 days in toxic, 9 days in sea water	0.48 mg.
After 12 days in toxic, 11 days in sea water	0.50 mg.
After 12 days in toxic, 11 days in sea water	(0.48 mg.)*

* Batch of dead barnacles.

TABLE X

Changes in copper content of soft tissues during recovery from various exposures to toxic

Toxic: Cupric citrate 0.75 mg. Cu/l.
Test Animal: B. eburneus
Date: October 21–November 12, 1942
Basis: Copper content per gram of dry weight of soft tissue

	Treatment	Copper content	Condition
Block L	After 3 days in toxic	0.38 mg.	++
	After 10 days in sea water	0.31 mg.	+
	After 14 days in sea water	0.21 mg.	+
Block M	After 6 days in toxic	0.38 mg.	+
	After 7 days in sea water	0.36 mg.	+
	After 14 days in sea water	0.40 mg.	++
Block N	After 9 days in toxic	0.58 mg.	0+
	After 9 days in toxic	(0.39 mg.)	dead individuals
	After 7 days in sea water	0.43 mg.	+
	After 14 days in sea water	0.39 mg.	++

No barnacles were found to have been killed by the absorption of less than 0.19 mg. of copper per g. of dry weight—an amount which is ten or more times the normal content of the tissues. In many cases animals succumbed after about 0.4 mg. per gram of dry weight had been taken up by the tissues. In no case did the barnacles absorb more than 1.09 mg. of copper per gram of dry weight. Barnacles which had accumulated copper from toxic solutions to the extent of 0.5 mg. to 1.09 mg./g. dry weight, in some cases revived when replaced in fresh sea water and were still alive two or three weeks later. At the end of the period, when they had regained normal, or nearly normal, activity, they contained 0.3 to 0.5 mg. of copper per g. of dry

TABLE XI

Changes in copper content of soft tissues during recovery from exposures at high concentrations of toxic

Toxic: Block O—Cupric citrate 0.90 mg. Cu/l.
Block Q—Cupric citrate 0.75 mg. Cu/l.
Test Animal: B. eburnesus
Date: October 21–November 21, 1942
Basis: Copper content per gram of dry weight of soft tissue

	Treatment	Copper content	Condition
Block O	After 8 days in toxic	0.52 mg.	0+
	After 8 days in toxic	(0.48 mg.)	dead individuals
	After 8 days in sea water	0.42 mg.	0+
Block Q	After 9 days in toxic	0.53 mg.	0+
	After 9 days in toxic	(0.35 mg.)	dead individuals
	After 8 days in sea water	0.40 mg.	+
	After 15 days in sea water	0.35 mg.	++

weight. In most instances the barnacles eliminated copper from their tissues, especially in cases where a high concentration had been accumulated. Although the copper which had been absorbed was never entirely eliminated, and although the process was slower than for accumulation, the experiments show that barnacles have some ability to rid their tissues of copper.

Accumulation of copper in mussel tissue during poisoning and recovery

To determine the amount of copper taken up by the tissues of the mussel when subjected to copper solutions, groups of 25 mussels were placed in various concentrations of cupric citrate and were later transferred to fresh sea water after increasing periods of time. The media were renewed every day. The copper content of 20 fresh animals from the same source was assayed at the beginning of the experiment and batches of three to five animals were withdrawn from the various groups for copper assay after the indicated number of days during poisoning and during recovery (Table XII).

The initial copper content of the mussel tissue was 0.0109 mg. per gram of dry weight, roughly the same as for the barnacle. Since the wet weight of the soft parts of the mussel was similarly found to be about ten times the dry weight, it is

clear that the fresh mussel also contains many times the concentration of copper present in the sea water.

After the first day in the toxic media, the mussels in the more concentrated solutions had taken up six to eight times their original amount of copper, but in the weaker solutions, there was no consistent increase in copper content until after an exposure of three days. The group of mussels (Group 4) in the weakest solution had absorbed only about two and one-half times their initial copper content after five days—or less than half the amount the Group 1 mussels had absorbed in one day in a solution five times as strong. In addition, the mussels were found to be able to rid their tissues of copper effectively. One day's sojourn in fresh sea water was suffi-

TABLE XII

Changes in copper content of soft tissues of mussel during poisoning and recovery

Toxic: Cupric citrate
Test Animal: Mytilus edulis, 25 animals (5 cm. long) in each group
Date: August 19 to September 5, 1942
Basis: Copper content in milligrams per gram of dry weight of soft tissue

Exposure to toxic solution began August 19. The line marks the time when mussels were transferred to fresh sea water

Group	Concentration of Cu citrate mg. Cu/l.	No. of days in toxic	Content on indicated date							
			Aug. 20	Aug. 21	Aug. 22	Aug. 23	Aug. 24	Aug. 25	Aug. 26	Sept. 5
1	0.120	1	0.066	0.0196	—	—	—	—*	—	—
2a	0.082	1	—	0.0135	—	—	0.017	—	—	0.0085
2b	0.082	2	0.084	0.031	0.023	—	—	—	—	0.0077
3	0.049	3	0.0076	—	0.024	0.018	—	—	0.017	0.0100
4	0.027	5	0.0135	—	0.021	—	0.025	0.029	—	0.0130
Control	0	0	0.0109							

* Condition on August 25: Group 1—all dead
Group 2a—9 dead
Group 2b—10 dead
Group 3—3 dead
Group 4—4 dead

No further deaths to September 5.

cient to reduce the amount of copper in the mussels from the strongest solutions by more than 70 per cent. In cases where longer exposures were employed (although in lower concentrations) more time seemed to be required for the elimination of the copper—perhaps because it had penetrated to deeper tissues. At the end of about two weeks in sea water, the copper content of the mussel tissue was back to normal—a fact quite in contrast to the situation with the barnacle.

In spite of the fact that after one day in sea water the copper content of the Group 1 mussels had been reduced at least to the amount reached by the mussels in the weaker solutions, all the animals in this group died within five days. Some irreparable damage had been done by the single day's exposure to the high concentration of the toxic. In the lower concentrations only three to four animals died

despite exposures to the toxic for three to five times as long. It is possible that the mussels in the lower concentrations could eliminate copper from vital tissues as fast as it tended to enter.

Although the values obtained in this single experiment are somewhat irregular, two important differences are indicated by the results as compared with the results obtained with barnacles. At the same concentrations, the exposures which are lethal to mussels are considerably shorter than for barnacles. Similarly the mussels succumbed after accumulating amounts of copper in their tissues which were much lower than was the case with barnacles.

Tests on the Poisoning of Cyprids

During January, 1943, experiments were undertaken at Miami Beach, Florida, on the poisoning of barnacles in the cyprid stage. Especial interest centered on these observations because the cyprid is the stage in which the animal first attaches itself to the substratum. It is the opinion of many that once a barnacle becomes attached, it can be killed only with great difficulty and that even if it is killed, the shell does not drop off but remains firmly attached. Actually, attachment takes place in two steps: first, the attachment of the antennae of the cyprid, following which metamorphosis is begun; and second, the attachment of the base of the barnacle after metamorphosis has been completed.

Cyprids were obtained by suspending glass microscope slides in Biscayne Bay overnight. The cyprids were presumed to be those of *Balanus improvisus*, which is the commonest adult barnacle in the neighborhood. The slides were brought into the laboratory where the following details in the development of the cyprids were observed.

The cyprids are originally attached by their antennae. About 5 or 6 hours after attachment, the animal undergoes metamorphosis in the course of which the cyprid shell is moulted and the animal emerges as a more or less round body still adhering to the substratum by the original attachment which now appears as the center of the convex base of the animal.

As development proceeds the base of the animal, which was at first freely movable, tends to flatten and to be pressed against the substratum. About 24 hours after metamorphosis the base is completely flattened with relatively sharp edges. A pulsation of the central parts of the animal has begun by this time, and the operculum may open, but no cirri are extended. The base is not caused to move by the activity of the animal, as before, but any part of it, except the original central point of attachment, is easily dislodged by pressure with a needle.

After another 24 hours, the cirri may be extended and filter actively, and the main area of the base begins to adhere to the substratum. At first the base merely gives the impression of being sticky; later it becomes attached with sufficient firmness so that it cannot be dislodged without tearing the tissue. Not until the third day or later does hard material (presumably calcium carbonate) begin to appear in the base.

The attachment of the cyprid by its antennae takes place quickly, and the original attaching structure remains in existence at least until calcification begins. The attachment of the base of the newly metamorphosed barnacle is a slow process which provides only weak adhesion at first but which finally supplies a permanent and

TABLE XIII

Effect of different concentrations of cupric citrate in preventing the metamorphosis of barnacle cyprids attached to glass slides

Species: probably Balanus improvisus

Concentration mg. Cu/liter	Number of cyprids exposed	Number of cyprids which metamorphosed
116.	6	2 (moult incomplete)
58.	6	3 (moult incomplete)
23.	4	4
9.7	3	3
4.9	2	2
0.93	11	11
0.47	9	9
0.23	5	5
0.12	9	9

firmly cemented calcareous structure. Poisoning of the barnacle could theoretically be accomplished either in the attached cyprid stage or as a newly metamorphosed animal before the calcification of the base.

The susceptibility of the cyprid to poisoning was investigated by placing cyprids attached to glass slides in solutions of cupric citrate (Table XIII) and of mercuric chloride (Table XIV). In the case of the copper solutions, the cyprids metamorphosed successfully in concentrations as great as 23 mg. Cu/l and solutions as strong as 116 mg. Cu/l were only partially inhibitory. Concentrations about 100 times greater than those ordinarily tolerated by adult barnacles, therefore, were not capable of killing the cyprids before they completed their metamorphosis.

In the case of the mercury solutions, although an inhibitory effect was observed at a lower concentration (16.6 mg. Hg/liter) than for copper, much higher concentrations of the toxic were required to prevent metamorphosis than would ordinarily kill adult barnacles.

Failure to kill the cyprid may be due to an unusually high resistance of the animal to poison in this stage or to the relative shortness of the period between attachment and metamorphosis, or both. From these results it is probable that the metamorphosis of the attached cyprid cannot be stopped by any concentration of toxic which could practically be obtained from a paint.

TABLE XIV

Effect of different concentration of mercuric chloride in preventing the metamorphosis of barnacle cyprids attached to glass slides

Species: probably Balanus improvisus

Toxic solution mg. Hg/liter	Number of cyprids exposed	Number which metamorphosed
83.	5	0
16.6	6	3 (moult incomplete)
3.3	6	6
0.66	9	9
0.17	7	7
0.09	10	10

The susceptibility to poisoning of the newly metamorphosed barnacle was tested in a similar fashion by placing slides bearing animals in this stage in solutions of cupric citrate (Table XV). Much lower concentrations of copper were required to kill the newly metamorphosed barnacle than was necessary to prevent the cyprids from carrying out their metamorphosis. The relation between killing time and concentration is roughly of the same order of magnitude as observed for adult barnacles at Woods Hole. There is some indication, however, that the newly metamorphosed animals are slightly more resistant, as concentrations of 0.23 mg. Cu/1 to 0.47 mg. Cu/1 were required to kill in five days in contrast to concentrations of only 0.14 to 0.30 mg. Cu/1 for the older animals.

Although recently metamorphosed barnacles survived for several days in concentrations of cupric citrate of 0.12 mg. Cu/1 or lower, their development was very materially retarded. In the stronger solutions the base remained in the rounded

TABLE XV

Relation of killing time to concentration of cupric citrate in barnacles which had just completed metamorphosis

Species: probably Balanus improvisus

Concentration mg. Cu/liter	Killing time days
9.7	1
4.9	1–2
0.97	3
0.93	3–4
0.47	5
0.23	5
0.20	7
0.12	10

condition, and in concentrations down to at least 0.20 mg. Cu/1 the base never became calcified nor rigidly cemented to the substratum, although it might become flattened and adhesive.

In a small number of tests reported subsequent to this investigation by Pyefinch and Mott (1944), free swimming cyprids of *Balanus balanoides* were killed in 24 hours by 0.5 to 1.0 p.p.m. copper from cupric sulphate. Metamorphosis was completed in concentrations up to 7 p.p.m. but after metamorphosis the barnacles were killed in 3 days by 0.5 p.p.m. Mercury from mercuric chloride was found to be more toxic than copper. These results agree very satisfactorily with ours.

At still lower concentrations of copper, in the present experiments, a complete change in the effect of the metal evidently comes about, for development was accelerated. Animals in the lowest concentration tested, namely 0.06 mg. Cu/1, reached the stage of active filtering with their cirri in less than 48 hours after metamorphosis, although the controls did not attain this stage until about 2 days later. It appears that a small amount of copper acted as a stimulant to development, and that larger amounts of copper retarded development even though they may be insufficient to kill the animals outright.

Discussion

Adult barnacles, and perhaps young stages also, can absorb ten to possibly one hundred times as much copper as their tissues normally contain without apparent injury. They can eliminate a certain amount of this material also. Therefore, when copper is used as a toxic in an antifouling paint, a substance is employed which can be taken up with impunity in relatively large quantities by the barnacle and to a lesser extent by the mussel. The animals can recover from the effects of this poison, to a certain extent at least, when again bathed by fresh sea water.

In some cases mussels which had received a lethal dose of poison remained in an apparent healthy condition for several days after being replaced in fresh sea water before dying. No such latent period was observed in the experiments with barnacles.

Possibly related to the facts just considered is the indication that the action of copper as a poison is not so much the direct destruction of some tissue or vital material in the barnacle as a general retardation of life processes. In the case of the adult barnacle, subjection to copper solutions causes a slowing and eventual cessation of the filtering activity of the cirri. In the case of the newly metamorphosed stages, not only is movement inhibited but development is seriously retarded as well. Much more satisfactory as a poison would be some substance which struck directly and irreversibly at some vital point in the animal and which could not be eliminated from the tissues. With such an ideal poison, barnacles which received only small doses, due to unavoidable dilution, would eventually be killed when a lethal amount of the material had accumulated. We have shown that with copper, sublethal doses may possibly stimulate development and certainly may allow subsequent recovery. Evidence that copper may stimulate the attachment of larvae has been reported by Prytherch (1934) for the oyster and by Miller (1946) for *Bugula*.

The relative vulnerability of the various steps in the attachment and growth of barnacles has been revealed by this investigation. The original idea that barnacles could be eliminated from a ship's hull only by preventing their initial attachment is partly right and partly wrong. As explained above, the attachment actually takes place in two steps. There is little promise of using poison successfully to prevent the first step, namely the attachment of the cyprid by its antennae, since this apparently takes place in a matter of minutes. However, we should not lose sight of the very important, but as yet unrealized, possibility of finding a substratum toward which the cyprid would display an avoiding reaction or to which the cement of the antennae would not adhere.[7]

Our tests have demonstrated the practical impossibility of preventing by poison the metamorphosis of the cyprid or of killing it during the process. This conclusion is substantiated by the results of Pyefinch and Mott (1944) and is in agreement with their statement: "Settlement and metamorphosis can take place on a paint in other respects anti-fouling though death of the barnacle occurs after metamorphosis." Similarly, at the other end of the life cycle, after the adult barnacle has attained any considerable size, it can be poisoned only with great difficulty for the reasons already discussed.

[7] In certain tests under laboratory conditions, Pyefinch and Mott (1944) found that cyprids failed to settle in concentrations of copper as low as 0.03 p.p.m.

There remains, however, the possibility of effectively preventing the second step in the attachment process, namely the permanent cementing of the base of the newly metamorphosed animal to the substratum. Although a long exposure to copper or a high concentration is necessary to kill the barnacle outright at this stage, moderate concentrations of poison tend to retard development and possibly to prevent the formation of the calcareous base, or the cement for this base, with the result that the young barnacle never succeeds in establishing a firm attachment, and hence is eventually displaced. The newly metamorphosed stage, therefore, appears to be the most vulnerable to copper poisoning.

The indication is, then, that copper paints act by preventing attachment but do so chiefly during the second step of the process through interference with the final cementing of the base of the metamorphosed barnacle to its substratum.

Summary

1. Direct tests were performed on the concentrations and exposures of a variety of metallic salts necessary to kill barnacles. The toxicities of mercury, cupric citrate, cupric tartrate, cupric salicylate, and cupric aminobenzoate were found to be slightly less than the toxicity of basic cupric carbonate. The toxicity of silver is about equal to that of basic cupric carbonate, but the toxicity of zinc is very much less.

2. The rate of killing of barnacles by cupric citrate is proportional to the concentration of the toxic over the range tested.

3. An extremely high concentration of copper or of mercury salts was necessary to prevent the metamorphosis of cyprids attached to glass plates. The results show the difficulty of preventing the *initial* attachment of cyprids, or their metamorphosis, by the use of copper paints.

4. Moderate concentrations of cupric citrate seriously retard the development of the newly metamorphosed barnacles and prevent the second step in attachment, namely, the formation of the cemented calcareous base.

5. Exposure of the newly metamorphosed barnacles to very low concentrations of cupric citrate accelerated development beyond that of the normal animals.

6. The soft tissues of adult barnacles normally contain a much higher concentration of copper than does sea water. When placed in solutions of cupric citrate, barnacles absorbed more than 100 times the normal copper content of the tissues. In no case were barnacles killed by the absorption of less than 0.19 mg. of copper per gram of dry weight—more than 10 times the normal content. In some cases barnacles which had absorbed 0.5 mg. to 1.09 mg./g. from toxic solutions revived when replaced in fresh sea water.

7. It was demonstrated that when replaced in fresh sea water, barnacles can eliminate from their tissues as much as two-thirds of the copper which has been absorbed from toxic solutions.

8. Mussels are more sensitive to poisoning by cupric citrate than barnacles. When exposed to copper solutions, mussels take up copper more rapidly than do barnacles, and when replaced in fresh sea water, they eliminate it from their tissues more rapidly and extensively. In many cases in which a considerable portion of the copper was eliminated, the mussels nevertheless succumbed subsequently.

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